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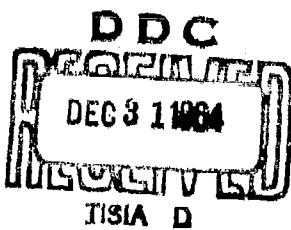
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TECHNICAL MANUSCRIPT 154

GROWTH CHARACTERISTICS
OF PSITTACOSIS GROUP AGENTS
IN HUMAN DIPLOID CELLS

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GROWTH CHARACTERISTICS OF PSITTACOSIS GROUP AGENTS
IN HUMAN DIPLOID CELLS

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ABSTRACT

Cultivation of human embryonic diploid cell strains (HDCS) and their use in preparing poliovirus vaccines were investigated by Hayflick and co-workers in 1962. The possible applications of these methods of virus cultivation to preparation of improved psittacosis vaccines have been investigated. A comparison was made of the growth and morphological development of two agents of human pneumonitis (strains Borg and SF) and one agent of psittacosis (strain 6BC) in one cell line (L cells) and several commercially available HDCS, including lung, skin and muscle, kidney, and whole embryo strains. Growth of the three agents was supported by each of the diploid cell strains in all combinations that were tested, although the three agents were not tested in each strain. Results obtained by growing the agents in HDCS were essentially the same as those obtained in the L cell line. A cytopathic effect (CPE) described as floating cellular debris, rounding of cells, holes, and a granular appearance throughout parts of the cell monolayer was observed in all cell cultures and with each of the agents used. The two strains (SF and Borg) of the human pneumonitis agent differed in growth pattern, CPE observed in cell cultures, and the type of inclusion bodies formed in human lung cells. The SF agent resembled more closely the 6BC strain of the psittacosis agent. Comparative titers, cytopathology, and morphological development of the agents in HDCS are discussed.

GROWTH CHARACTERISTICS OF PSITTACOSIS GROUP AGENTS
IN HUMAN DIPLOID CELLS

Considerable interest has developed in the possible use, for virus production, of cell strains derived from human embryos which can be maintained in tissue culture through many but not an indefinite number of passages.¹⁻³ The human diploid cells exhibit those characteristics usually applied to "normal" or "primary" cell cultures, including the normal human diploid karyotype. The human diploid cell strains, when inoculated into the hamster cheek pouch or terminal human cancer patients, do not develop local growths which have the morphological characteristics of malignancy.¹ The storage of surplus cells from the early passages of well standardized strains would make the strains available continuously, and in addition, it would be possible to rule out the presence of extraneous viruses so that such a strain could be used for the production of human virus vaccines. The current considerations of practicability, the unusually broad human virus spectrum of these cells^{2,3} and the potentialities of the diploid strains make this cell system an attractive substrate for vaccine preparation. Their use in the preparation of attenuated poliovirus vaccines has been reported.⁴

The present investigations were initiated to determine the susceptibility of several commercially available human embryonic diploid cell strains to various agents of the psittacosis group. Such cells may be used to develop immunizing antigens for man against these agents. In this communication, comparative titers, cytopathology, and morphological development of three psittacosis group agents in human diploid cell strains are presented and discussed.

Table I shows the agents of the psittacosis group and the human embryonic diploid cell strains used in these studies. Earle's mouse L fibroblast cell line was available in our own laboratories.

The human diploid cell strains were grown in 250-ml bottles in antibiotic-free medium consisting of Eagle BME prepared with Hanks' BSS and supplemented with ten per cent calf serum and two millimoles glutamine. The pH of the medium was 7.3. When cell cultures were held beyond the fourth day, the serum concentration of the maintenance medium was reduced to three per cent. The growth medium for the L cells consisted of antibiotic-free medium 199 supplemented with ten per cent calf serum and was used at pH 7.3 to 7.5. The serum concentration in maintenance medium was three per cent.

Confluent cell sheets were infected with a 10^{-2} dilution of infected ^{50%} tissue culture seed of the psittacosis group agent, incubated at 37°C for one

TABLE I. PSITTACOSIS GROUP AGENTS AND HUMAN EMBRYONIC DIPLOID CELL STRAINS USED IN THE PRESENT EXPERIMENT

I. PSITTACOSIS GROUP AGENTS

A. Psittacosis - 6BC strain

B. Human pneumonitis - Borg and SF strains

II. HUMAN EMBRYONIC DIPLOID CELL STRAINS

A. Flow Laboratories, Inc., Rockville, Md.

1. Lung strains 2004, 2008, 2037 and WI-38
2. Skin and Muscle 1009
3. Kidney 4031
4. Whole Embryo 5037

B. Microbiological Associates, Inc., Bethesda, Md.

1. Lung strain, M-7

hour, and washed three times with medium. Cultures were incubated at 37°C and the medium was replaced daily. Infectivity was determined by titrating serial tenfold dilutions of the agent in beef heart infusion broth via the yolk sac route in seven-day embryonated eggs. LD₅₀ calculations were made by the method of Reed and Muench.

Growth of the psittacosis group agents in L cells was studied initially in order to have a basis for later comparisons when these agents were grown in human diploid cell cultures. The daily release of the 6BC, SF, and Borg agents into supernatant fluids during the first passage of these microorganisms in L cells and the progression of cytopathic changes are shown (Figure 1).

The 6BC agent showed a slow but fairly steady rise in titer through Day 7 when the first passage experiment was terminated. The progressive cytopathology, which became definite on the fourth day, showed a similar pattern. Although the data are not shown here, the leveling out of the titer was more pronounced during the second passage. The SF agent showed a more rapid rise in titer during the first three days, and on the third day the release of the agent into supernatant fluid leveled out. The progression of the cytopathic effect (CPE) correlated with the rise in titer. The most

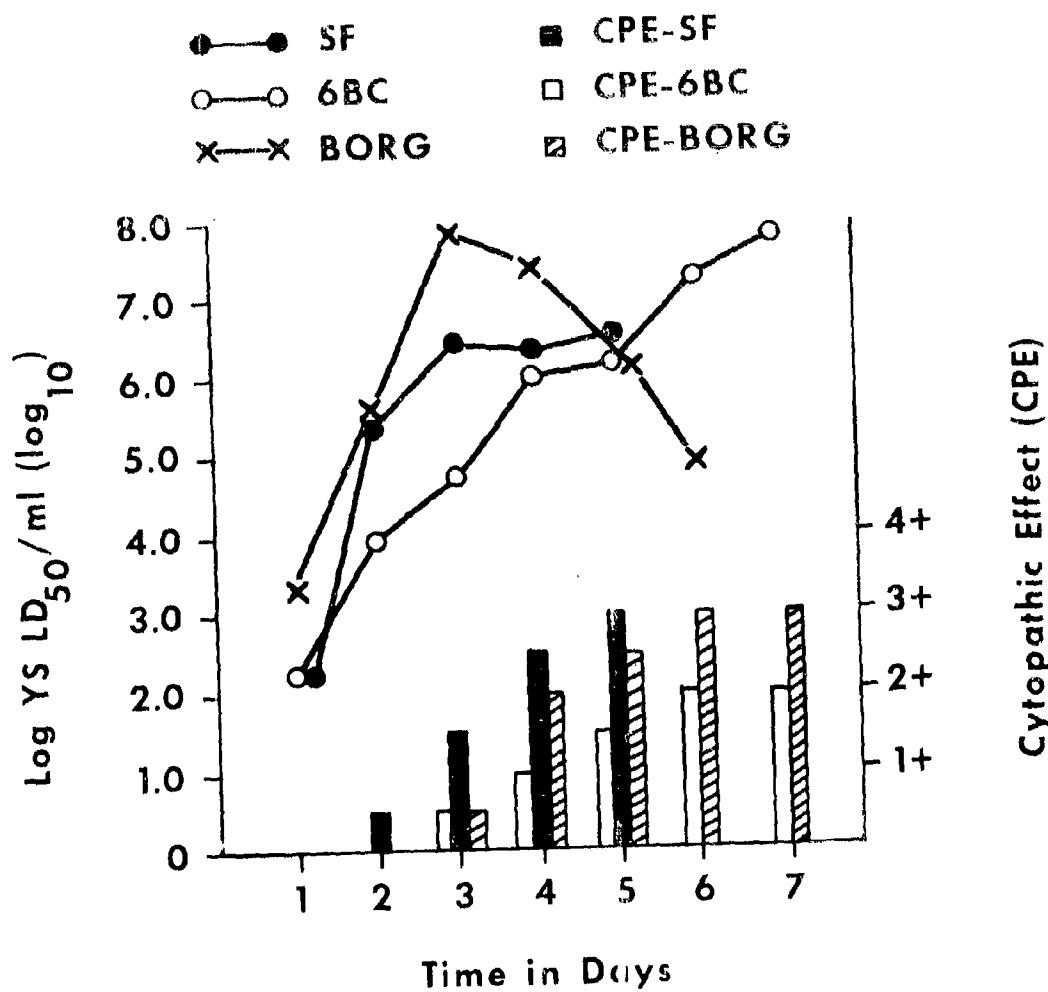


Figure 1. Growth Curves and Cytopathic Effects of SF, 6BC, and BORG Agents in L Cells.

rapid rise in titer occurred with the Borg agent, which reached a maximum titer of $10^{7.8}$ YS LD₅₀/ml on the third day at which time the titer of the agent declined rapidly. Although the data are not shown for Days 7 and 8, the titers were $10^{2.3}$ and less than $10^{0.7}$ YS LD₅₀/ml respectively. The CPE was first observed on Day 3 and continued to progress until Day 6. At the termination of the experiment, those cells still remaining attached to the glass surface were rounded and very granular in appearance. The SF and Borg agents showed almost identical results in the second passage.

Table II shows comparative titers of the SF agent in three human diploid cell strains. With the lung strain 2004, release of the agent proceeded to maximum titer, which remained constant. No significant increase in maximum titer was observed in second or third passages of this agent in the cell strain. A single passage of this agent in lung strain M-7 was essentially the same as the growth pattern observed with cell strain 2004. With the skin and muscle strain 1009, the release of the SF agent into the supernatant fluid followed the same growth pattern as was observed with the lung strains.

TABLE II. COMPARATIVE TITERS OF HUMAN PNEUMONITIS AGENT SF GROWN IN HUMAN DIPLOID CELL MONOLAYERS

Tissue Source	Cell Strain Number	Passage Number in Cells	Log YS LD ₅₀ /ml on Indicated Days						
			1	2	3	4	5	6	7
Lung	2004	1		4.7	5.3	6.1	6.5	5.7 ^a	5.9
		2		4.7	5.7	6.7 ^a	6.1	6.3	5.6
		3		4.7	5.9	6.1	5.9	5.3	5.4
	M-7	1	2.7		4.6	5.1	6.5	6.4	
Skin and muscle	1009	1		5.3	5.1	6.3 ^a	6.3	6.2	
		2		5.4	6.6	6.6			

a. Indicates supernatant fluids used as inoculum for subsequent passages.

Figure 2 compares the growth of the 6BC and Borg agents with the growth of the SF agent in lung strain 2004. The results compared favorably with the data shown previously for growth of these agents in the L cells. The 6BC agent showed a rapid rise in titer during the first three days and thereafter the release of the agent was slower. Although the data are not shown here, the leveling out of the agent titer was more pronounced in the second passage. A rapid rise followed by a sharp decline in agent titer was observed with the Borg agent. The SF agent showed a slower rise in titer and the titer declined or leveled out after the maximum titer of $10^{6.5}$ YS LD₅₀/ml. occurred.

Figure 3 shows the CPE observed with the three psittacosis group agents grown in the lung strain 2004. In general, the CPE began with the appearance of increased floating cellular debris in the supernatant fluids, rounding of cells and a granular appearance throughout parts of the cell monolayer. Within a day or two, areas of cell sloughing or holes in the monolayer were observed and the intensity of granulation increased. The most intense CPE was observed with the Borg agent; however, the CPE never progressed to the point where all the cells came off the glass.

Additional human diploid cell strains used for the propagation of the Borg and 6BC agents were whole embryo, kidney, lung strains WI-38, 2008, and 2037 (Table III). Comparative titers of the two agents in cell monolayers of these tissues showed that the maximum titers observed with these agents were not significantly different from the results obtained by propagating these two agents in lung strain 2004. A CPE was observed with each of the tissues and did not differ from the cytopathology discussed previously for the human lung strain 2004.

Additional studies were undertaken to examine the morphological appearance of the SF, 6BC and Borg agents in diploid human embryonic lung culture 2004. Cells were grown on cover slips contained in Leighton tubes and were infected with an undiluted agent seed grown in tissue culture. Cover slips were removed daily, stained by the May-Grunwald-Giemsa method, and examined microscopically for the presence of inclusions.

Figure 4 shows the morphological appearance of the Borg strain of the human pneumonitis agent. The arrows point to inclusion bodies. During the first 24 to 50 hours the inclusion bodies were small, rounded and tightly packed. A more diffuse, or spreading type of inclusion body was observed at 18 hours.

Figure 5 shows the inclusion bodies seen with the 6BC strain of the psittacosis agent at 72 and 96 hours post infection. The inclusions appeared as tightly packed, compact bodies.

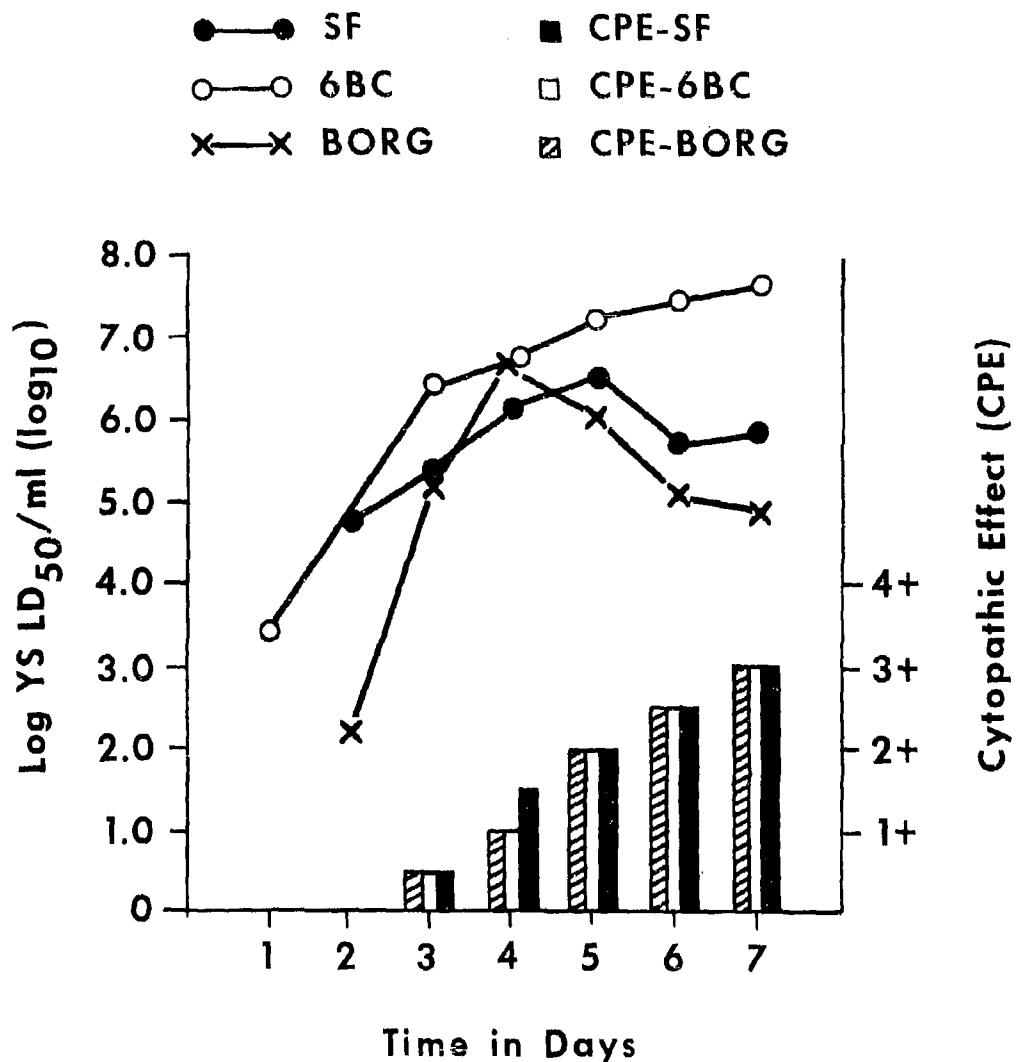


Figure 2. Growth Curves and Cytopathic Effects of SF, 6BC, and Borg Agents in Human Diploid Cell Strain 2004.

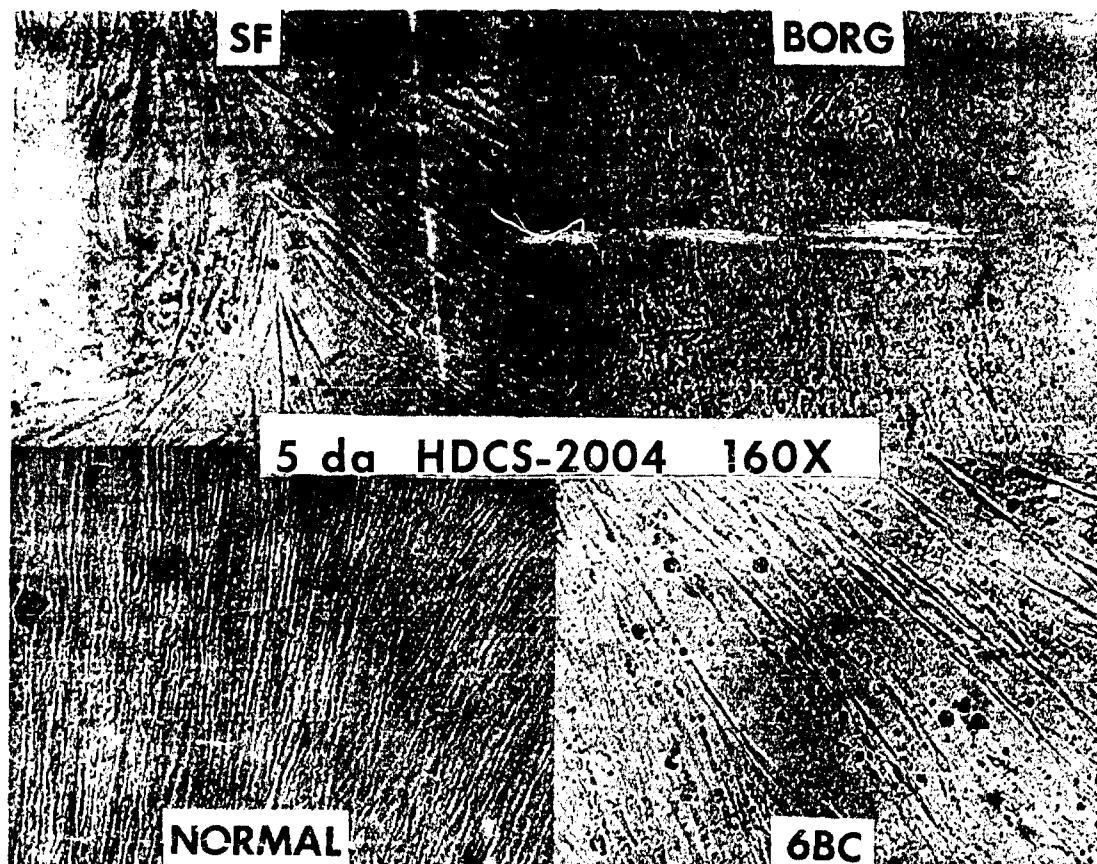


Figure 3. Cytopathology of SF, Borg, and 6BC Agents in Cell Monolayers of Human Diploid Cell Strain 2004.

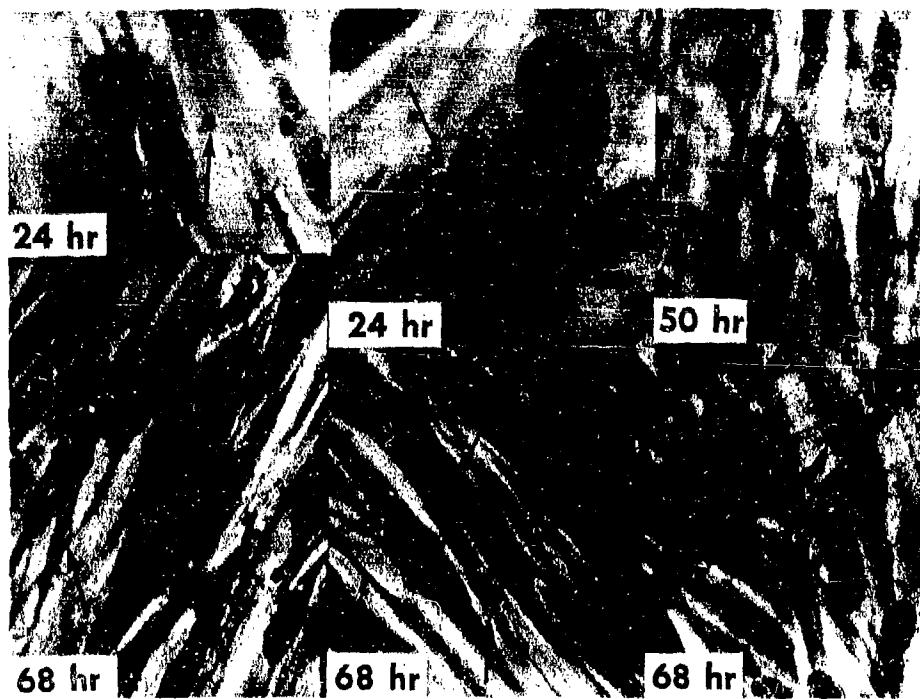


Figure 4. Morphological Appearance of Borg Agent in Human Diploid Cell Strain 2004. 1000X



72 hr

96 hr

Figure 5. Morphological Appearance of 6BC Agent in Human Diploid Cell Strain 2004. 1200X

TABLE III. GROWTH OF BORG AND 6BC AGENTS IN VARIOUS HUMAN DIPLOID CELL STRAIN MONOLAYERS

Tissue Source	Cell Strain Number	Agent	Log YS LD ₅₀ /ml on Indicated Days								
			0	1	2	3	4	5	6	7	8
Whole embryo	5037	Borg ^a /Borg ^a	<1.7 2.0	<1.7 >4.7	4.3 6.5	6.3 7.3	7.6 6.7	7.1 >5.7			
Kidney	4031	Borg	1.7	2.6	6.3	6.9	6.3				
Lung	WI-38	Borg	<2.7	1.7	6.3	7.3	6.4	6.3			
Lung	2037	6BC	2.0	<2.7	>5.7	6.5	8.1	8.1	7.6		
Lung	2008	6BC	2.5	3.3	5.3	5.3	6.1	6.1	7.1	7.3	7.1

a. Second passage.

Since investigations on the growth of the SF strain of the human pneumonitis agent in tissue culture have not appeared in the literature, it was of interest to compare the morphological development of the SF agent with the Borg strain of the human pneumonitis agent. Previously, we had noted that the growth pattern and the CPE development observed with the SF agent in tissue cultures differed from that seen with the Borg agent.

The photomicrographs in Figure 6 show the development of the SF agent. Small, round, compact inclusion bodies were observed during the early stages of development. These bodies continued to increase in size with time until in some instances (as shown in the 92-hour photograph) the inclusion body or bodies completely filled the entire cytoplasm.

Figure 7 shows three inclusion bodies of the SF agent at a greater magnification.

These studies indicate that the SF agent resembled more closely the 6BC strain of the psittacosis agent, especially in regard to the compact type of inclusion body. In further studies it would be of interest to compare the virulence of these agents for tissue culture with their virulence for animals.

In conclusion, a comparison was made of the growth and morphological development of three agents of the psittacosis group in one cell line and several human diploid cell strains. These investigations showed that all diploid cell strains supported the growth of one or more of the three agents of the psittacosis group. The results obtained by the growth of the agents in diploid cell

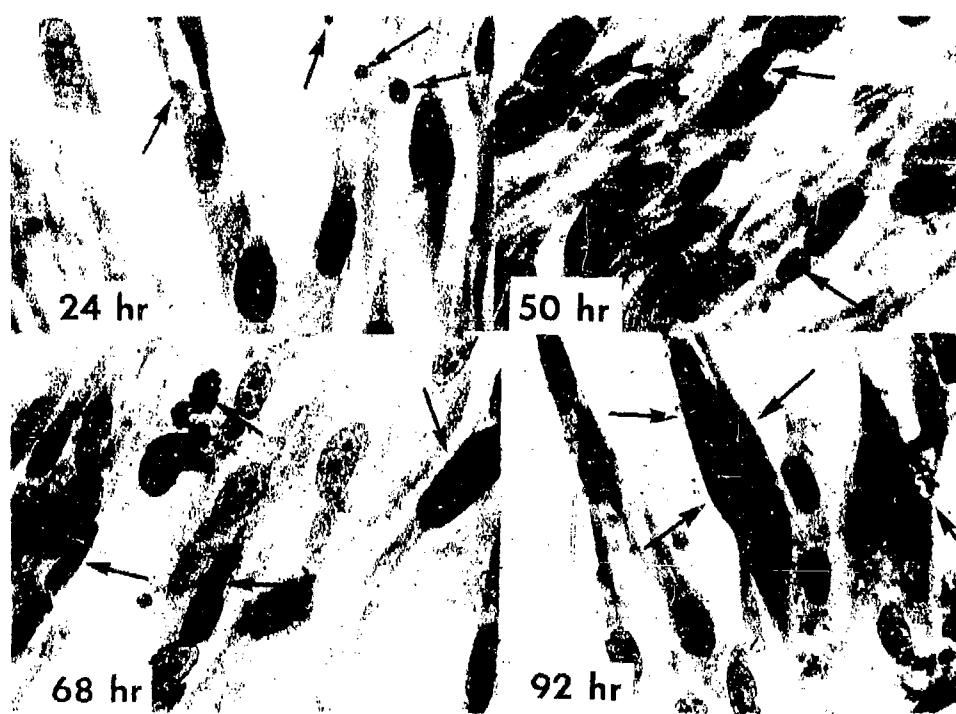


Figure 6. Morphological Appearance of SF Agent in Human Diploid Cell Strain 2004. 1000X



Figure 7. Inclusion Bodies of the SF Agent at 68 Hours in Human Diploid Cell Strain 2004. 2700X

strains were essentially the same as those obtained in the cell lines. The ability of the agents of psittacosis and human pneumonitis to grow in the diploid strains suggest that it may be of value to explore further the possible utilization of these cells in the preparation of vaccines for the psittacosis group.

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